INBREEDING EFFECTS ON PHYSIOLOGICAL RESPONSES TO CHRONIC HYPOXIA IN MICE (*Mus musculus*)

Jennifer Irene Berting

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Approved by:

Advisory Committee

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Chair

Accepted by:

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Dean, Graduate School

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ABSTRACT

Changes in complex phenotypes, such as exercise tolerance, are often mediated by the co-adjustment of many components. For example, hypoxic exercise tolerance (HET) is typically enhanced by the myriad physiological adjustments that accompany acclimation to hypoxia. Further, exercise tolerance is strongly influenced by genetic background, but the effect of genetic variation is largely unknown. The present study sought to examine the consequence of reduced genetic variation, in the context of inbreeding, and its effect on the relationship between hypoxic exercise tolerance (HET), maximal aerobic capacity (VO_{2max}), and myoglobin concentration ([Mb]) in *Mus musculus*. I hypothesized that the increased homozygosity found in inbred strains would lead to reduced phenotypic plasticity, resulting in more variable responses to chronic hypoxia. In addition, I hypothesized that HET and VO_{2max} would be positively correlated. Hypoxia led to decreases in body mass as well as increases in HET and hypoxic VO_{2max} in all strains. While there was no significant effect of breeding on HET, the change in VO_{2max} and the cost of exercise following hypoxic acclimation were greater among inbred than outbred strains. The response of [Mb] to hypoxia was generally greater among inbred than outbred strains as predicted, but the differences were mostly non-significant. HET and VO_{2max} were positively correlated, but [Mb] was not correlated with either of these variables. The consistent influence of inbreeding on body mass, VO_{2max}, and cost of exercise suggest an underlying consequence of genetic uniformity. While there was some support for the hypothesis that reduced phenotypic plasticity in inbred strains leads to a more variable response to hypoxia, on the whole the results were not consistent with this view.

ACKNOWLEDGEMENTS

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INTRODUCTION

Insufficient oxygen delivery to tissues can occur for many reasons including disease, increased oxygen demand during exercise, or environmental conditions such as high altitude (Ferretti et al., 1990; Fisher et al., 1992). Chronic exposure to hypoxia (at least several days) causes a suite of changes in organisms that promote oxygen delivery to tissues, and the hypoxic response often includes increases in hemoglobin concentration, hematocrit, 2,3-bisphosphoglycerate (BPG) concentration, capillarity, vasodilation, and myoglobin concentration ([Mb]) (Baumann et al., 1971; Banchero, 1987; Terrados et al., 1990; Guilleman and Krasnow, 1997; Hochachka, 1998; Marshall and Davies, 1999; Hammond et al., 2001; Ernst, 2003). These and other cell, tissue, and whole organism level changes during hypoxia help to preserve the capacity for exercise under hypoxia, which is often positively associated with the whole body maximal rate of oxygen consumption (VO_{2max}) (Bassett and Howley, 2000).

There is growing evidence that exercise capacity and the responses to chronic hypoxia that influence exercise vary according to genetic background (Bouchard et al., 1986; Matheson et al., 1991; Holden et al., 1995; Hochachka et al., 1996; McCall and Frierson, 1997; Howlett et al., 2003). The heritability of endurance and a physiological phenotype that promotes endurance has been clearly demonstrated in rodents. Mice (*Mus musculus)*, originally of the Hsd:ICR strain, have been artificially selected for high or low voluntary wheel running and VO_{2max} was 6-10% higher in high running lines than in low running lines during both normoxic (Swallow et al. 1998; Rezende et al. 2006; Garland and Kelly, 2006) and hypoxic exercise (Rezende et al., 2005b). In a separate series of studies, rats (*Rattus norvegicus,* of the N:NIH stock) have been bidirectionally selected for forced treadmill running capacity (Koch and Britton 2001). VO_{2max} was found to be ~20% higher in high endurance capacity lines than in low endurance capacity

lines during normoxic and hypoxic exercise (Gonzalez et al., 2006). These results indicate that VO_{2max} is positively correlated with selection for habitual activity as well as forced exercise in rodents. Other studies investigated the effect of genetic background on aspects of endurance in the absence of selection. For example, Lightfoot et al. (2001) found strong evidence of a genetic contribution to exercise capacity among 10 strains of inbred mice in forced treadmill running and later (Lightfoot et al., 2004) similar results in daily wheel running activity among 13 strains of inbred mice. Additionally, McCall and Frierson (1997) demonstrated large differences among a variety of inbred and recombinant inbred strains of mice in hypoxic exercise tolerance (HET).

The previous work of McCall and Frierson (1997) utilized two inbred strains of mice: BALB/cByJ (C) and C57BL/6J (B6). The mice were exposed for 8 weeks to either hypobaric hypoxia (1/2 atmospheric PO_2) or normoxia (sea level PO_2) after which they underwent forced treadmill exercise under hypoxia to determine HET. Mice that were acclimated to normoxia displayed moderate interstrain differences in HET. However, after acclimation to hypoxia, there were large differences in HET between the strains. Subsequent studies revealed a number of hypoxia-induced modifications that may aid in oxygen delivery to tissue as well as changes in muscle that may modify oxidative capacity and substrate utilization (Adams, 2002; Ernst, 2003; Ludeke et al., 2004; Sarkar, 2005). A particularly intriguing finding was that hypoxia acclimation had dramatically different effects between strains at multiple levels of biological organization. In B6 mice, whole animal endurance (HET), VO_{2max}, and several components of the respiratory cascade responded to hypoxia with large increases. In contrast, C mice had smaller changes or no response to hypoxia (McCall and Frierson, 1997; Ernst, 2003; Sarkar, 2005). The clearest illustration of the variation in the hypoxic response was in [Mb], which differed between strains and tissues (skeletal and cardiac muscle) in both magnitude and

direction of change (Ernst, 2003). Myoglobin is an oxygen-binding hemoprotein found in cardiac and oxidative skeletal muscle that increases oxygen solubility in cytoplasm via reversible binding (Wittenberg, 1970; Wittenberg and Wittenberg, 1975; Cole, 1982; Conley et al., 2000; Garry et al., 2000; Tilakaratne et al., 2002; Ordway and Garry, 2004). Therefore, it is able to act as the final mediator of oxygen flux from the atmosphere to the mitochondria in the oxygen delivery cascade, and it is the only component of the cascade that is intracellular (Wittenburg, 1970; Taylor et al., 1986; Garry et al., 1998; Godecke et al., 1999; Nitta et al., 2003; Duteil et al., 2004).

The large and variable responses of HET, VO_{2max}, and [Mb] to hypoxia in C and B6 mice may be related to inbreeding. Due to their genetic uniformity, inbred strains are frequently used models because they can help elucidate genetic causes for variations in complex phenotypes like aerobic capacity (Troxell et al., 2003; Koch and Britton, 2001; Barbato et al., 1998). However, genetic uniformity may have consequences such as a limited range of phenotypic plasticity, a subject that has been debated for half a century since Lerner's (1954) suggestion of heterozygote superiority. This concept was defined as the superior "buffering capacity" of heterozygous individuals that enables the organism to overcome environmental perturbations and develop more closely to the phenotypic optimum for the species, termed developmental homeostasis (Lerner, 1954; Merola, 1994). Since then, the detrimental effects of high genetic uniformity to physiological fitness (inbreeding depression) and, likewise, the improved fitness due to increased genetic variation (heterosis or hybrid vigor) has been shown in many studies (Lynch, 1977; Pierce and Mitton, 1982; Mitton et al., 1986; Teska et al., 1990; Margulis, 1998; Fowler and Whitlock, 1999; Campbell et al., 2007). For instance, increased heterozygosity has been shown to be positively correlated with metabolic efficiency (measured as work done per O_2 consumed),

longevity, growth rate, fecundity, and overall developmental stability (Prud'homme, 1984; Koehn and Gaffney, 1984; Mitton and Grant, 1984; Merola, 1994; Hildner and Soule, 2004).

A lack of phenotypic plasticity may account for the variable responses to hypoxia in the C and B6 inbred strains. Since myoglobin is the final mediator of oxygen flux to the cell, changes in [Mb] may indicate the efficacy of the upstream responses to chronic hypoxia in maintaining a balance between oxygen supply and demand at the tissue. Therefore, if phenotypic plasticity is limited for some components of the oxygen delivery cascade due inbreeding, it may necessitate relatively large fluctuations in [Mb] in response to hypoxia. In contrast, outbred strains may be better able to co-adjust upstream components of the cascade in response to hypoxia. Limitations of individual components of the respiratory cascade may constrain the available range of VO_{2max}, which may, in turn, limit the responsiveness of endurance exercise capacity to changing environmental conditions. Weibel et al. (1991) examined such co-adjustment in the respiratory system of several sedentary and active mammals (dog, goat, horse, and steer) to test the hypothesis of symmorphosis, which posits that structural design is matched to functional demand. They found that within the oxygen delivery cascade, co-adjustment that was consistent with the concept of symmorphosis took place at all steps (blood, heart, capillaries, and mitochondria), except at the lungs. Thus, the higher heterozygosity of outbred strains may permit a symmorphotic matching of structure and function that results in smaller changes in [Mb] in response to acclimation to hypoxia than in inbred strains.

Since VO_{2max} integrates the multiple components of the oxygen delivery system, including myoglobin, it represents a functional limitation of the cardiovascular system (Hill and Luptin, 1923; Bassett and Howley, 2000) and provides a physiological context for interpreting [Mb]. The ability to limit the decrease in VO_{2max} under hypoxia has long been used as an index

of the capacity for acclimation to hypoxia (Young et al., 1990; Ferretti et al., 1997; Calbet et al., 2003a; Calbet et al., 2003b). Ernst (2003) and Sarkar (2005) examined hypoxia induced changes in HET, VO_{2max}, and [Mb] in the C and B6 mice but found no clear relationship between the variables. However, Duteil et al. (2004) found that aerobic capacity of human skeletal muscle, estimated from the recovery kinetics of phosphocreatine (PCr), was positively correlated with [Mb]. It is possible that the lack of a relationship between [Mb], VO_{2max}, and HET in inbred strains is a result of genetic uniformity.

The present study sought to examine the effect of breeding on the relationship between the hypoxic responses of whole animal performance (HET), an integrated index of whole animal aerobic capacity (VO_{2max}), and a component of the respiratory cascade ([Mb]). Three additional inbred strains and four outbred strains were combined with the data collected previously by McCall and Frierson (1997), Ernst (2003), and Sarkar (2005) for the C and B6 mice. I tested the hypothesis that HET and VO_{2max} were positively correlated, and that the response of HET, VO_{2max}, and [Mb] to chronic hypoxia would be more variable in inbred than outbred strains.

MATERIALS AND METHODS

Animal Maintenance

Inbred strains C3H/HeJ, DBA/2J, and FVB/NJ were acquired from the Jackson Laboratory, Bar Harbor, Maine. Outbred strains CD-1 and CF-1 were obtained from Charles River Laboratories, Wilmington, MA, while Swiss Webster (SW), and Black Swiss Webster (BSW) strains were obtained from Taconic Farms, Inc., Germantown, NY. All mice were housed in polycarbonate cages in a laminar flow hood at 23 +/- 1^{o} C under a 12:12 hour light-dark cycle until 8 weeks of age. Animals were provided Agway 3000 Mouse Chow and water *ad libitum*. At 8 weeks of age, mice were arbitrarily split into the two treatment groups. Half remained in the original colony (normobaric, sea level PO_2) for an additional 8 weeks, and half were moved to a hypobaric chamber that maintained half atmospheric PO_2 (Altitude Fitness, LLT, Littleton, CO) for 8 weeks. Cages were continually flushed with air and temperature was maintained between 22-24^{o}C. Food and water were available *ad libitum* in the chamber.

Hypoxic Exercise Tolerance

HET was determined based on the protocol outlined previously by McCall and Frierson (1997). After 8 weeks of acclimation to either normoxia or hypoxia, the mice were exposed to a "forced-choice" exercise test, which was accomplished using a homebuilt enclosed treadmill. Mice were weighed and then placed in the treadmill compartment, which was flushed continuously with a normobaric hypoxic gas mixture. The gas was premixed 10.47% O_2 in N_2, the same PO_2 as that in the hypobaric chamber, and monitored by an oxygen analyzer (Model S-3A/II, AEI Technologies, Naperville, IL). Flow to the treadmill was regulated by a digital mass flow control unit (Series 100 Smart-Trac, Sierra Instruments, Monterey, CA).The mice were held

in the compartment with the treadmill stationary for 5 min, during which time they were free to explore the compartment and to discover the 10 x 10 cm bar grid at the base of the treadmill that delivered a mild 0.15 mA scrambled current when two bars were touched. After the acclimation period, the treadmill belt, set to a 15^{o} incline, was gradually increased in speed until a velocity of 40 cm/s (1.44 km/h) was reached. The mice ran on the treadmill at this speed until the endpoint, HET, which was determined as the time when the mouse spent 4 consecutive seconds on the shock grid.

VO_{2max} and Cost of Exercise

Normoxia and hypoxia acclimated mice were transferred to an enclosed modular treadmill designed for physiological measurements in mice (Columbus Instruments, Columbus, OH). The sealed treadmill had a volume of 2 L and was flushed continuously with a premixed normobaric hypoxic gas (10.47 ± 2% O_2 in N_2). Air in the chamber was rapidly turned over by a small fan at the head of the treadmill. The air flow rate was maintained at 50 ml/s with a Series 100 Smart-Trak digital mass flow controller (Sierra Instruments, Monterey, CA). Air was drawn from both the inflow line and from the treadmill chamber by a dual channel R-2 flow controller (AEI Technologies, Naperville, IL). The sample air from the treadmill had all water vapor and CO_2 removed by passing it through a column of drierite and ascarite. The air then passed to a dual channel S-3 A/II differential oxygen analyzer (AEI Technoogies, Naperville, IL). Under these conditions, 98% of the air in the treadmill chamber was exchanged after 3 min. For this reason, all VO_2 measurements were made 3 min after a change in belt speed. Upon entrance to the treadmill, mice were allowed to explore the treadmill compartment for 10 min and VO_2 was recorded while the treadmill was stationary after this 10 min time period. The treadmill was then

started at 10 cm/s and after 3 minutes, VO_2 was recorded. Belt speed was then increased incrementally by 5 cm/s every 3 min and VO_2 was recorded until VO_2 failed to increase with increasing speed and/or the animal failed to keep pace with the treadmill. A subjective assessment of run quality was performed, where a rating of 1 to 3 was determined; 1 being poor and 3 being excellent. Mice that did not run with the belt continuously and instead attacked the shock grid or attempted to escape for segments of the trial, received scores of 1. Mice that ran with the belt, but were not able to run well, were given a score of 2, and mice that ran fully until the end of the trial without any inconsistencies were given a score of 3. Trials that scored a 1 were excluded from calculations.

Steady state VO_2 (ml O_2/min/kg) was determined using the equation of Bartholomew *et al*. (1981):

$$VO_2 = V(F_{IO2} - F_{EO2})/1 - F_{IO2}$$

Where V = flow of CO_2-free dry air, F_{IO2} = fractional concentration of O_2 in the incurrent air, and F_{EO2} = fractional concentration of O_2 in the excurrent air. Additionally, cost of exercise ((ml O_2/min/kg)/(cm/s)) was calculated by dividing VO_{2max} by the lowest belt speed at which VO_{2max} was attained. The C and B6 data collected previously by Sarkar (2005) were not included in these calculations because a different treadmill belt speed acceleration schedule was used to determine VO_{2max}.

Myoglobin Concentration

Mice were euthanized via inhalation of 100% CO_2. For spectrophotometric measurement of [Mb], the left and right gastrocneumius, soleus, extensor digitorum longus (EDL), and the right ventricle (RV) were dissected while submerged in mouse Ringer's solution (117 mM NaCl,

4.5 mM KCl, 2.5 mM $CaCl_2$, 1.16 mM $MgSO_4$, 20 mM $NaHCO_3$, pH 7.4) and continually aerated with a 95% O_2 / 5% CO_2 gas mixture. Following dissection, muscles were scored on a color scale, generated using Adobe Photoshop (version 7.0), that encompassed the color range of the tissues. This allowed for a comparison between pigmentation and [Mb] measured spectrophotometrically in order to further support the spectrophotometric measurements. The tissues were then flash-frozen in liquid nitrogen and stored at -85^{o}C.

Myoglobin concentrations were determined based on techniques previously described by Reynafarje (1963) and modified by Ernst (2003). Frozen tissues were thawed on ice, blotted dry, weighed, cut into ~1mm^3 pieces, and transferred to test tubes. Samples were diluted by a factor of at least 20 with 0.04 M phosphate buffer, pH 6.6. Smaller tissues required larger dilutions in order to obtain adequate sample volumes. Tissues were homogenized on ice (PowerGen model 125 homogenizer, Fisher Scientific, Hampton, NH) via 3 cycles of 10-s bursts. Tissues were then sonicated for three 10-s bursts using a Fisher Scientific model 60 sonic dismembrator. Samples were transferred to 20-mL high-speed centrifugation tubes and centrifuged for 50 min using a Beckman J2-21M/E centrifuge (Beckman, Fullerton, CA) at 29,100 x *g* at 0^{o} C. After centrifugation, the supernatant was transferred to 15 mL centrifuge tubes and bubbled with CO for 8 min. To ensure full reduction of myoglobin, 0.03 g of sodium hydrosulfite was added to the sample and mixed using a Maxi Mix II (Barnstead/Thermolyne, Dubuque, IA) vortex mixer. The sample was bubbled with CO for an additional 2 minutes. Immediately after bubbling, approximately 0.07 mL of the sample was transferred to a quartz microcuvette. The concentration of myoglobin was then determined based on the difference in absorbances at 538 and 568 nm using a Pharmacia Ultrospec 4000 spectrophotometer (Pharmacia Biotech, Cambridge, England) according to Reynafarje (1963).

Statistical Analysis

Data previously collected for inbred C and B6 mice by Ernst (2003) and Sarkar (2005) were included in the analysis to more fully evaluate the influence of inbreeding. Body mass was analyzed using two-way analysis of variance (ANOVA) to test for significant effects of breeding and treatment, strain and treatment, or an interaction between the main effects. The two-way approach was necessary because the strain and breeding effects were confounded, and the data were therefore not amenable to a full 3-way ANOVA model. HET, VO_{2max}, and cost of exercise were analyzed by two-way analysis of covariance (ANCOVA) to test for significant effects of breeding and treatment, strain and treatment, or interactions between variables. Body mass was included as a covariate in these analyses because it explained some of the variation in the HET, VO_{2max}, and cost of exercise data. [Mb], on the other hand, had no relationship with body mass and so was analyzed by ANOVA rather than ANCOVA. Strain comparisons for each variable were made using two group T-tests, and P-values were adjusted to compensate for multiple tests according to the Bonferroni method. Correlation analysis was used to investigate the relationship between HET, VO_{2max}, and [Mb]. Results were considered significant if $p< 0.05$. All analyses were run with Statistical Analysis System (SAS) software (version 9) (SAS Institute, Cary, NC).

RESULTS

Animal body mass

The mean body mass of mice used in this study as well as from the study by McCall and Frierson (1997) are presented in Figure 1. Two-way ANOVA detected a significant effect of treatment (d.f. = 1, F = 49.14, $p<0.0001$) and breeding on body mass (d.f.=1, F=308.16, $p<0.0001$) but no interaction. Mice acclimated to hypoxia had a lower body mass than those acclimated to normoxia, and inbred mice were significantly lower in body mass than outbred mice. Two-way ANOVA also revealed a significant effect of strain (d.f.=8, F=52.47, $p<0.0001$) and an interaction between strain and treatment (d.f.=8, F=2.08, p=0.0367), and least squares analysis revealed that the lower body mass in mice acclimated to hypoxia was significant for all strains except C3H (p=0.3685), DBA (p=0.059), and BSW (p=0.6491).

Hypoxic Exercise Tolerance

Two-way ANCOVA, with body mass as a covariate, found a significant treatment effect (d.f.=2, F=50.66, $p<0.0001$) on HET, and all strains had higher HET following hypoxic acclimation, although this was not significant in C3H, C, and BSW mice (Figure 3). However, there was no effect of breeding or an interaction of breeding and treatment, indicating that inbreeding did not influence the magnitude of the difference in HET between normoxic and hypoxic acclimated mice. While overall, inbred strains had higher mean HET (22.46 ± 1.5 min) than outbred strains (11.95 ± 1.5 min), this difference was due almost exclusively to the high HET found in B6 and DBA mice. There was a significant strain (d.f.=8, F= 99.21, $p<0.0001$) and strain-treatment interaction (d.f.=8, F=24.09, $p<0.0001$) on HET, and intra-strain differences between normoxic and hypoxic acclimated mice are shown in Figure 3. DBA mice had the

highest HET values following acclimation to normoxia (33.5 ± 4.1 min) and were significantly higher than all strains except B6 mice, which also had high HET values (21.3 ± 1.1 min). Variation among strains was higher in hypoxia acclimated mice than in those raised in a normoxic environment. Most strains had an approximate 2-fold higher HET following hypoxic acclimation. DBA mice had the highest HET values (67.0 ± 2.0 min), which entailed a 3-fold higher HET after hypoxic acclimation, while SW mice had a 7-fold higher HET following hypoxic acclimation.

VO_{2max} and Cost of Exercise

Two-way ANCOVA, using body mass as a covariate, revealed a significant treatment effect on VO_{2max} (d.f.=1, F=195.92, $p<0.0001$), and all strains had a higher VO_{2max} after acclimation to hypoxia, as seen in Figure 3. However, the magnitude of difference between normoxic and hypoxic acclimation was significantly higher among inbred strains than among outbred strains, which was reflected by a significant breeding-treatment interaction (d.f.=1, F=33.86, $p<0.001$). Inbred strain VO_{2max} was on average 35.9 ± 2.6 $ml \cdot min^{-1} \cdot kg^{-1}$ higher after acclimation to hypoxia, while outbred strains were only 14.5 ± 2.6 $ml \cdot min^{-1} \cdot kg^{-1}$ higher. ANCOVA also revealed a significant strain effect (d.f.= 1, F=2.93, p=0.0047) and strain-treatment interaction (d.f.=1, F=6.86, $p<0.0001$) on VO_{2max}. The strain effect was due mostly to the higher VO_{2max} values in inbred strains after acclimation to hypoxia (mean=144.78±2.45 $ml \cdot min^{-1} \cdot kg^{-1}$) relative to outbred strains (122.27±2.54 $ml \cdot min^{-1} \cdot kg^{-1}$), as indicated by the higher F statistic for the breeding effect than for the strain effect. Additionally, two-way ANCOVA found significant effects of breeding (d.f.= 1, F=5.09, p=0.0257) and a breeding-treatment interaction (d.f.=1, F=10.19, p=0.0018) on cost of exercise (Figure 5), however, there was no

effect of treatment (d.f.=1, F=3.74, p=0.055). There were also significant strain (d.f.=6, F=3.03, p0.0085) and strain-treatment interactions on exercise cost (d.f.=6, F=2.54, p=0.0239).

Myoglobin Concentration

[Mb] in right ventricle, soleus, gastrocnemius, and EDL for all strains and treatments are presented in Figure 7. Muscles with lower [Mb] were notably paler in appearance upon dissection, and comparison of color scale assignments to [Mb] confirmed a high level of overall correlation (r^2=0.68, p<0.0001) as well as significant correlations within each muscle type (RV: r^2=0.111, p<0.0007; soleus: r^2=0.233, p<0.0001; gastrocnemius: r^2=0.113, p<0.0004; EDL: r^2=0.108, p<0.0006), which supports the accuracy of the spectrophotometric measurements. In most cases, the highest concentrations of myoglobin were found in the right ventricle with progressively lower concentrations in the soleus, gastrocnemius, and EDL respectively. The only deviation from this occurred in the C mice which had [Mb] in EDL that was slightly higher than in gastrocnemius.

Two-way ANOVA did not indicate a significant effect of treatment, breeding, or an interaction between the two in the RV (Figure 7). Treatment effects were not significant because most strains displayed little change in [Mb] following hypoxic acclimation, and in the two strains with large differences between treatments (C and SW mice), [Mb] changed in opposite directions following hypoxia acclimation. A significant strain effect (d.f.=8, F=6.07, p<0.0001) and an interaction between strain-treatment (d.f.=8, F=2.08, p=0.0432) was detected. The strain-treatment interaction was mostly due to the large change in [Mb] following hypoxic acclimation found in the C and SW mice (Figure 8A).

Significant treatment (d.f.=1, F=5.21, p=0.024) and breeding (d.f.=1, F=7.19, p=0.0083) effects were found for [Mb] within the soleus, although there was no significant interaction (Figure 7). Following hypoxic acclimation, all strains had either higher [Mb] or had no change in [Mb] with the exception of C mice, which had lower [Mb]. Inbred strains had higher mean [Mb] (0.380±0.019 g/100g tissue) than outbred strains (0.305±0.021 g/100g tissue). A significant strain effect was also found (d.f.=8, F=3.22, p=0.0024) as was a strain and treatment interaction (d.f.= 8, F=4.39, p=0.0001). The interaction was largely the result of variation in the inbred strains, where hypoxia induced relatively large changes in [Mb] that varied in magnitude and direction of change (Figure 8B).

There was no significant effect of treatment, breeding, or a breeding and treatment interaction on [Mb] in the gastrocnemius (Figure 7). Strain effects were also non-significant, however, a significant strain and treatment interaction was found (d.f.=8, F=2.02, p=0.049) and, again, it resulted primarily from the relatively large hypoxia induced variation in [Mb] in inbred strains.

A significant effect of treatment (d.f.=1, F=6.7, p=0.0105) and breeding (d.f.=1, F=4.84, p=0.029) were found in EDL [Mb], but no significant treatment and breeding interaction (Figure 7). A significant strain effect was found (d.f.=8, F=5.35, p<0.0001) as was a strain and treatment interaction (d.f.-8, F=2.67, p=0.0099). As in the soleus and gastrocnemius, strain and treatment interaction for [Mb] in the EDL resulted largely from hypoxia induced variation in inbred strains. These results are summarized in Figure 8, where the change in [Mb] in response to hypoxia can be seen to be generally greater in inbred than outbred strains.

Relationship between HET, VO_{2max}, and [Mb]

Correlation analysis revealed that mean HET and VO_{2max} were positively correlated, although there was large variation (r^2= 0.25, p=0.036) (Figure 9). In contrast, there was no significant relationship between HET and [Mb] (Figure 10) or VO_{2max} and [Mb] (Figure 11), although a positive correlation between VO_{2max} and [Mb] was nearly significant in the soleus (r^2=0.45, p=0.055). There were some significant correlations of [Mb] with HET and VO_{2max} among some individual groups of mice. Among hypoxic acclimated mice, HET was positively correlated with [Mb] in EDL (r^2 = 0.74117, p=0.0223) (Figure 10). However, among outbred strains, there was a strong negative correlation between HET and [Mb] under hypoxic acclimation in both the RV (r^2= -0.97027, p=0.0297) and gastrocnemius (r^2= -0.96705, p= 0.0329) (Figure 10). Under hypoxic acclimation in outbred mice, there was a significant positive correlation between VO_{2max} and RV [Mb] (r^2= 0.97, p=0.029) (Figure 11).

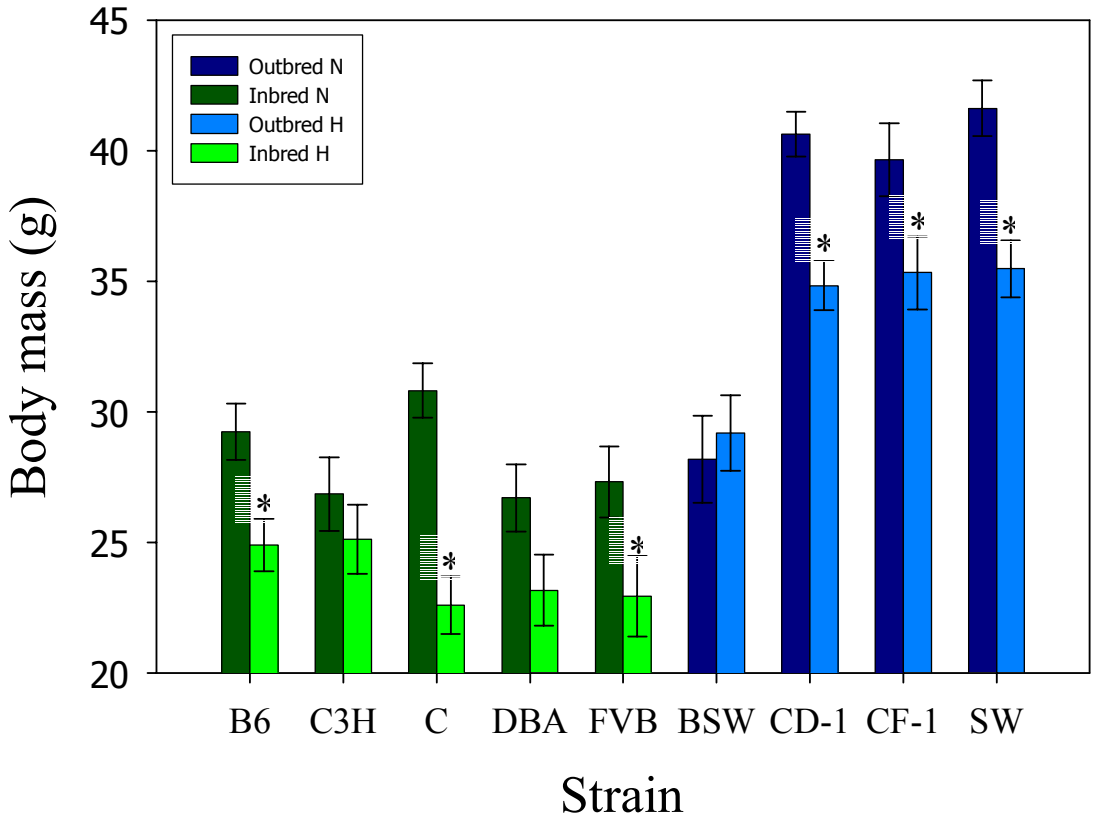


Figure 1. Animal body mass following acclimation to normoxia and hypoxia. Values shown are means ± SEM. An * indicates a significant difference between mice acclimated to normoxia and hypoxia. See text for additional results of statistical analyses.

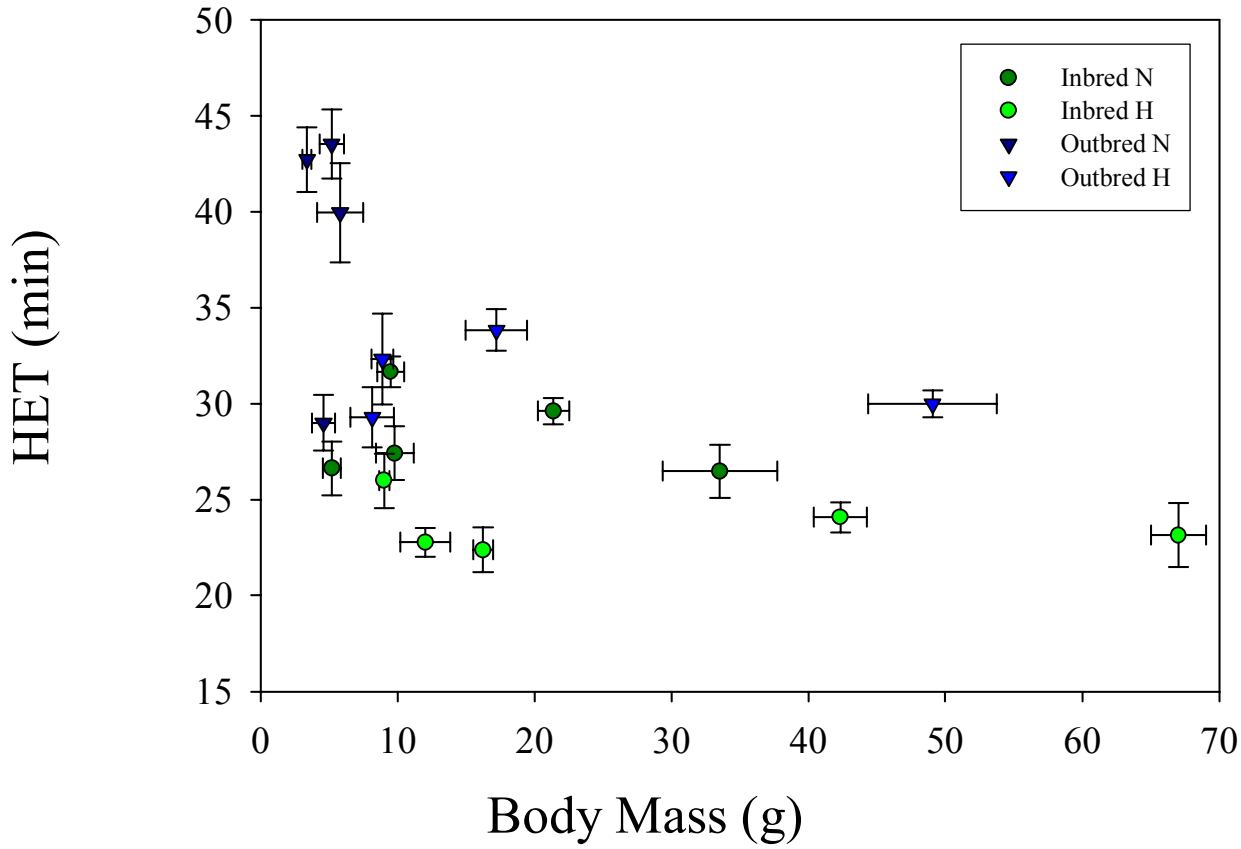


Figure 2. HET vs. body mass.

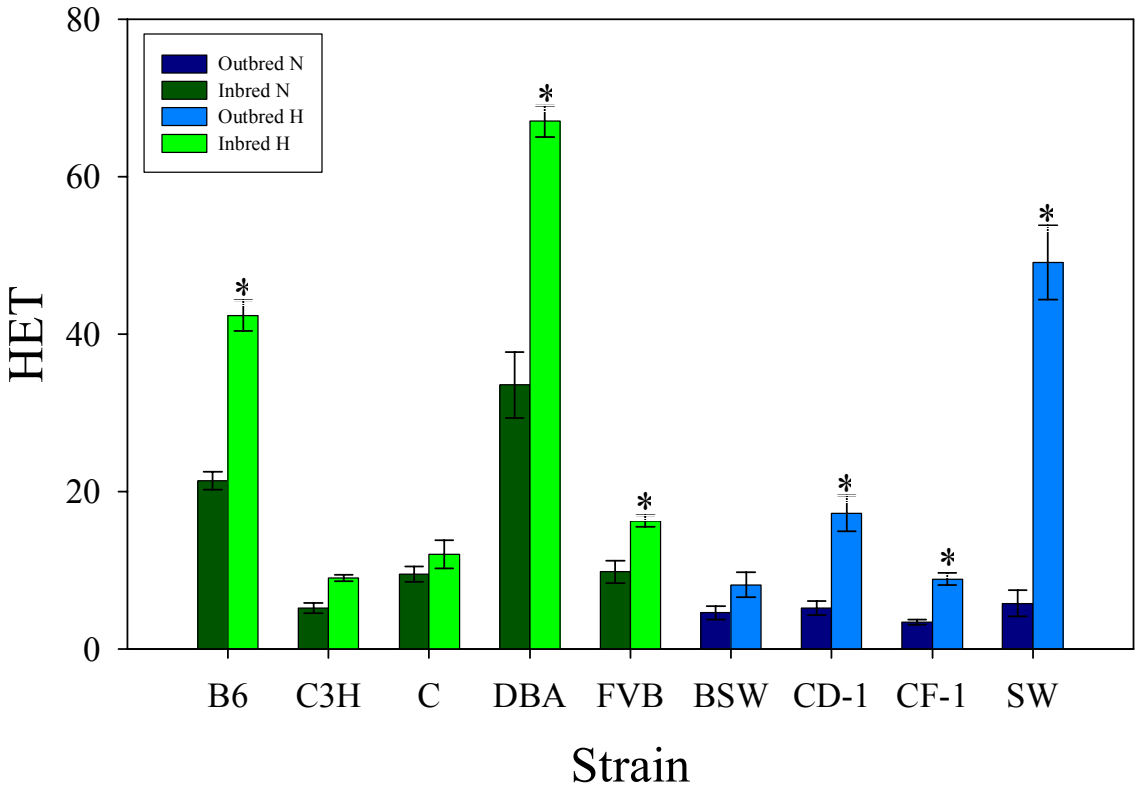


Figure 3. HET following acclimation to normoxia and hypoxia. All values shown are mean ± SEM. An * indicates a significant difference between mice acclimated to normoxia and hypoxia. See text for additional results of statistical analyses.

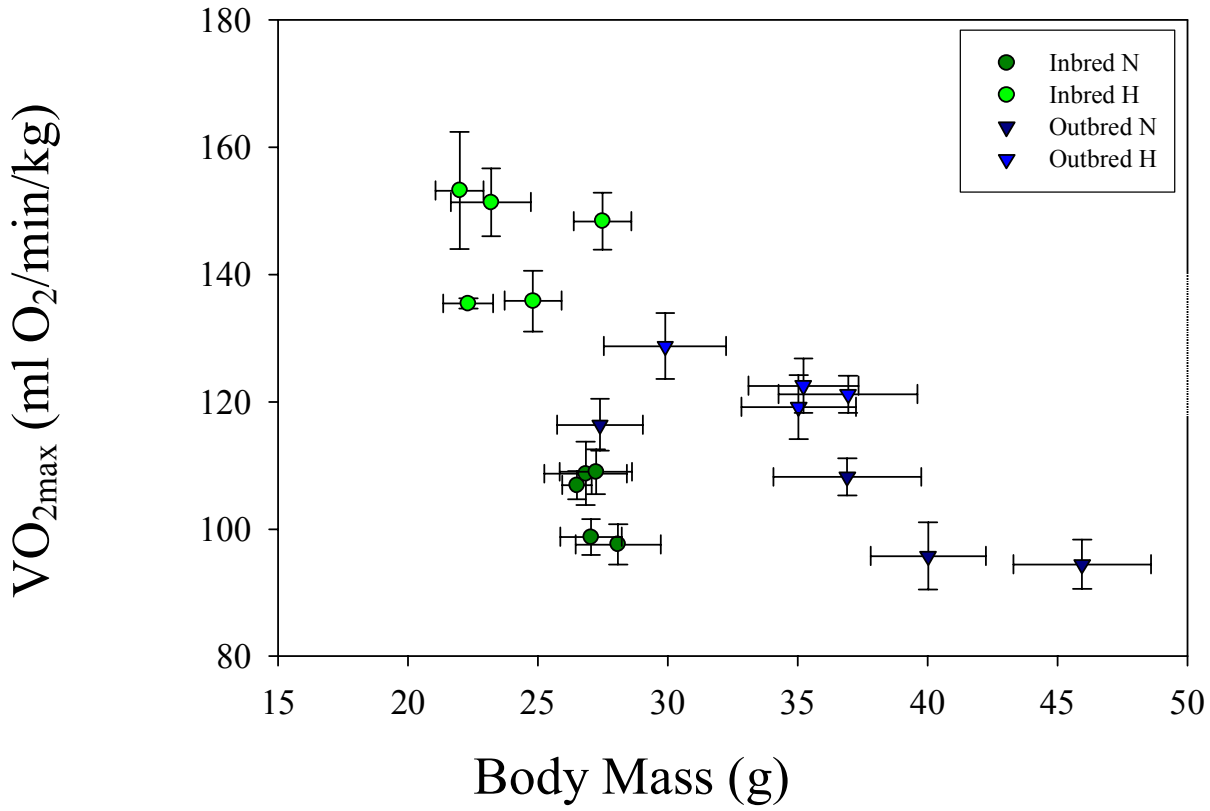


Figure 4. VO_{2max} vs. body mass.

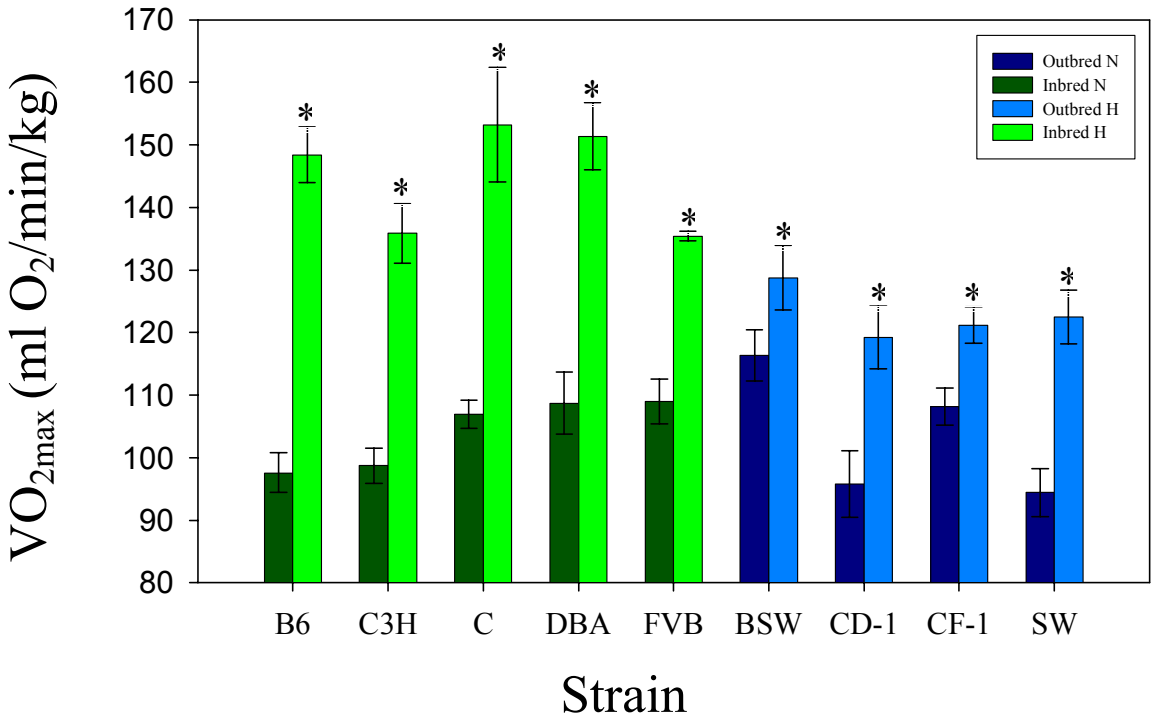


Figure 5. VO_{2max} following acclimation to normoxia and hypoxia. Values are means ± SEM. An * indicates a significant difference between mice acclimated to normoxia and hypoxia. See text for additional results of statistical analyses.

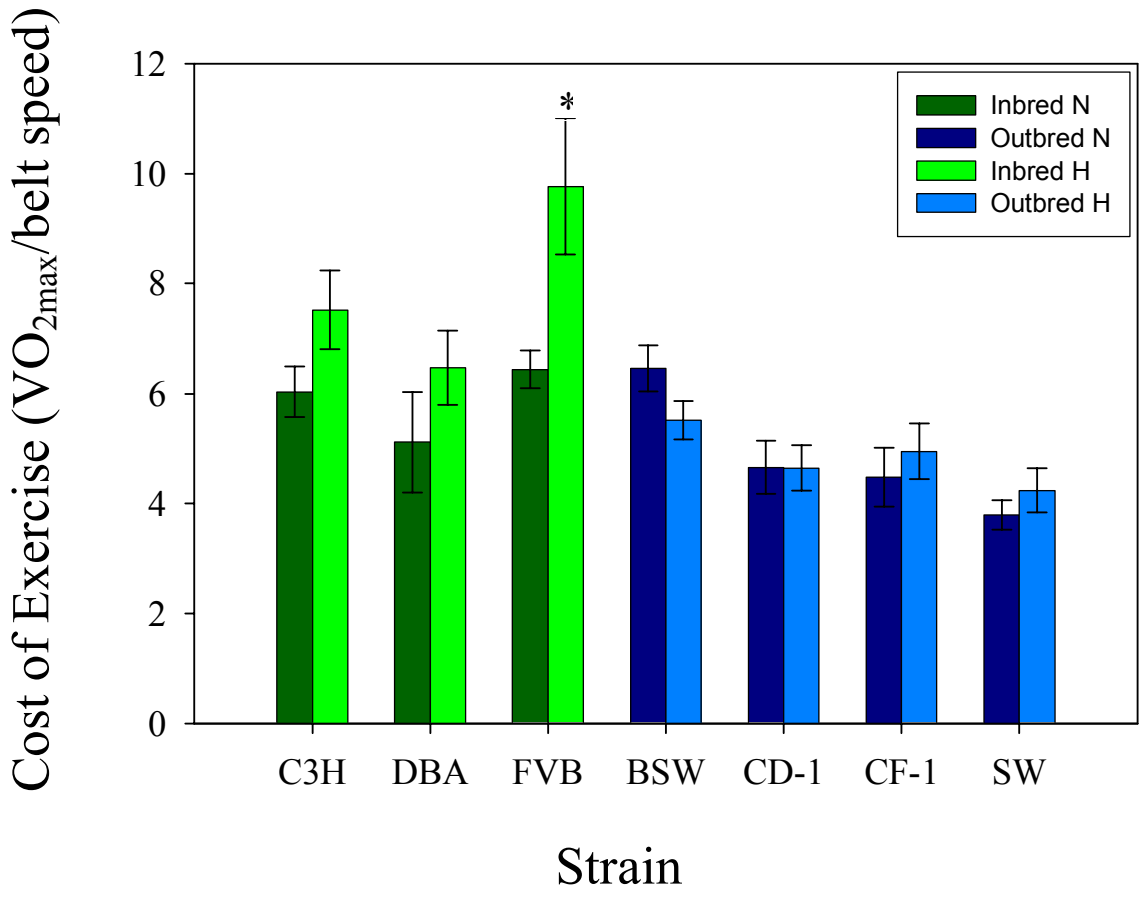


Figure 6. Cost of exercise in inbred and outbred strains following acclimation to normoxia and hypoxia. VO_{2max} has units of ml O_2/min/kg and belt speed has units of cm/s. Values are means ± SEM. An * indicates a significant difference between mice acclimated to normoxia and hypoxia. See text for additional results of statistical analyses.

Figure 7. [Mb] within the (A) RV, (B) soleus, (C) gastrocnemius, and (D) EDL in inbred and outbred strains following acclimation to normoxia and hypoxia. Values are means ± SEM. An * indicates a significant difference between mice acclimated to normoxia and hypoxia. See text for additional results of statistical analyses.

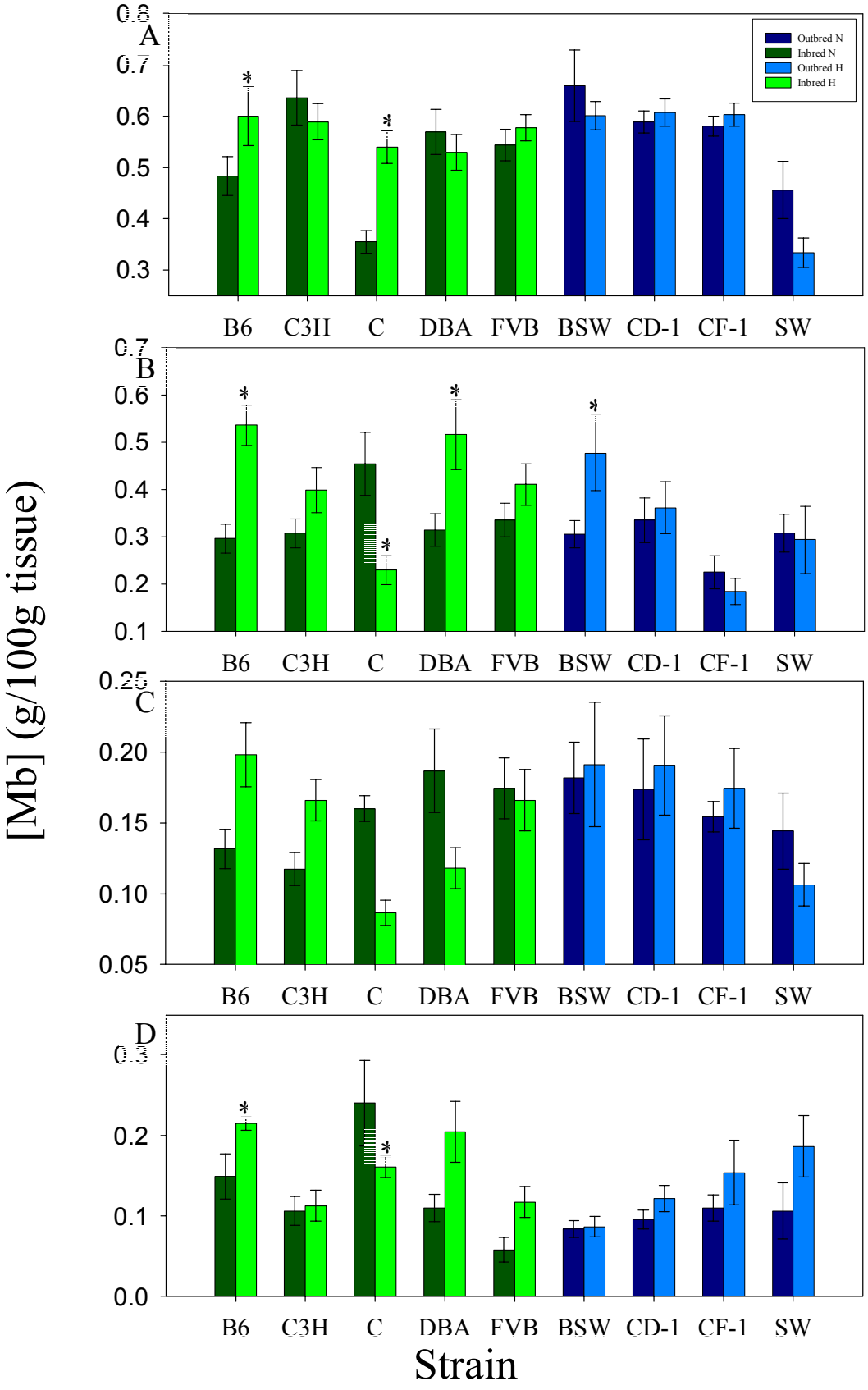
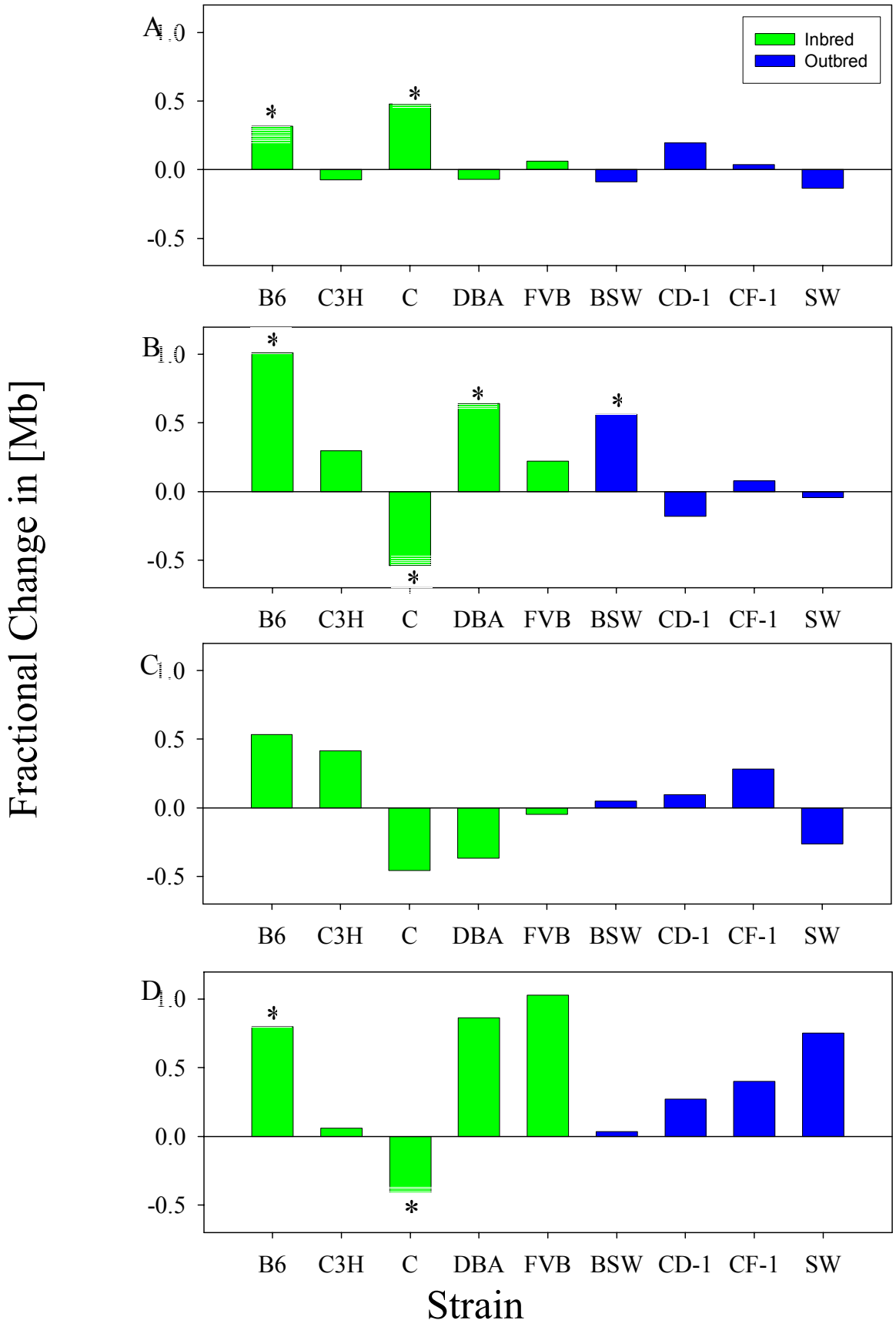

A
B
C
D
Outbred N
Inbred N
Outbred H
Inbred H
B6
C3H
C
DBA
FVB
BSW
CD-1
CF-1
SW
[Mb] (g/100g tissue)
Strain

Figure 8. Fractional change in [Mb] for the (A) RV, (B) soleus, (C) gastrocnemius, and (D) EDL in inbred and outbred strains following acclimation to normoxia and hypoxia. An * indicates a significant difference between mice acclimated to normoxia and hypoxia.


A
B
C
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Inbred
Outbred
1.0
0.5
0.0
-0.5
*
B6
C3H
C
DBA
FVB
BSW
CD-1
CF-1
SW
Fractional Change in [Mb]
Strain

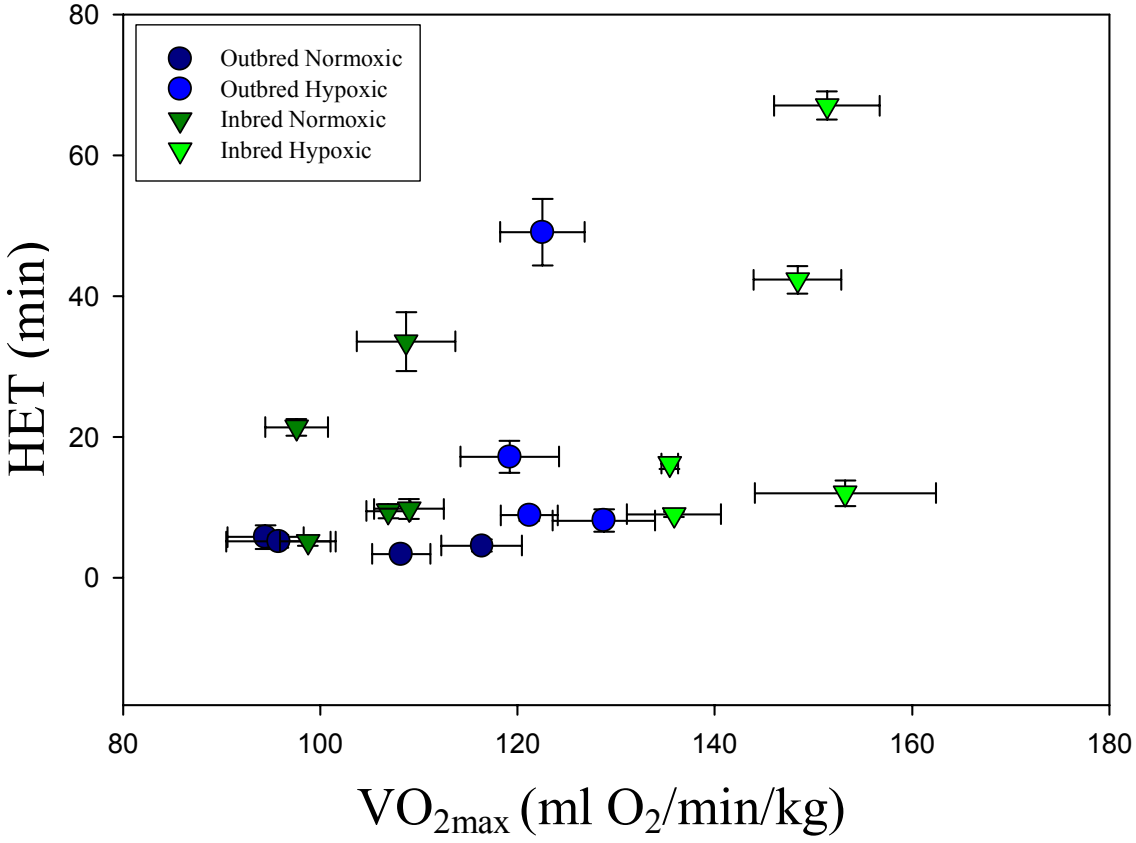

Figure 9. Relationship between HET and VO_{2max}. Values are means ± SEM. Individual data points correspond to different strains or acclimation treatment. There was a positive correlation between HET and VO_{2max} (see text for additional details).

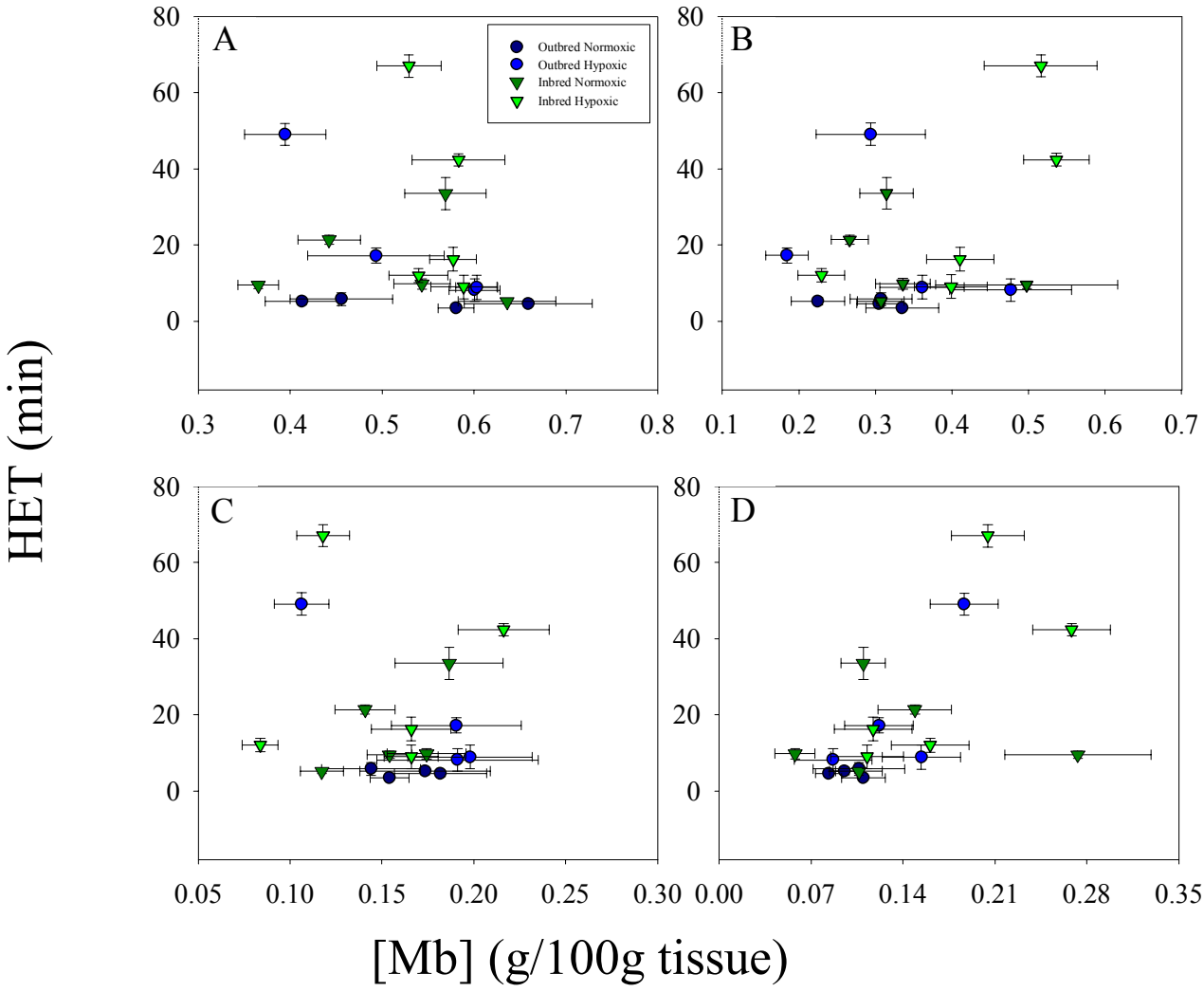

Figure 10. Relationship between HET and [Mb] in (A) RV, (B) soleus, (C) gastrocnemius, and (D) EDL. Values are means ± SEM. Individual data points correspond to different strains or acclimation treatments. There were no significant correlations when using all strains and treatments (see text for additional details).

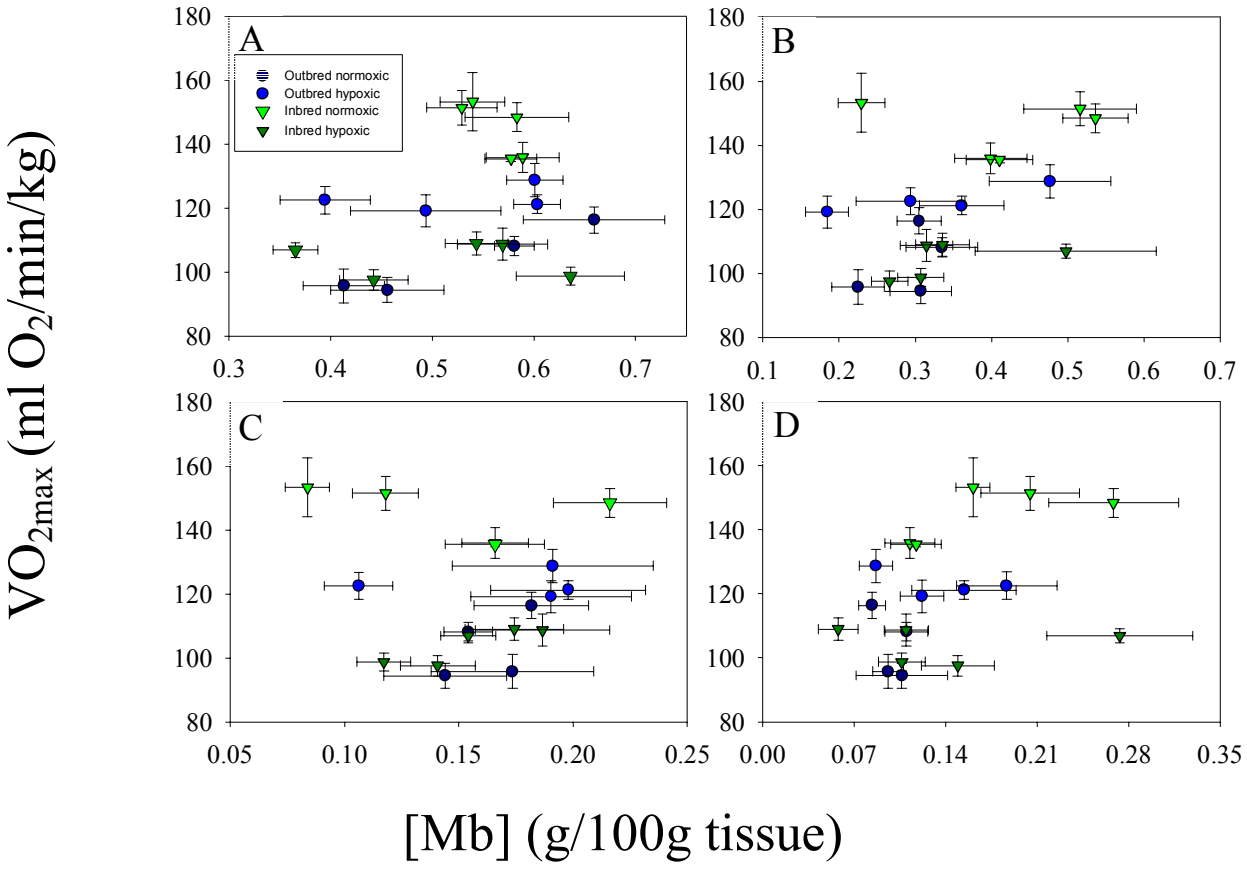

Figure 11. Relationship between VO_{2maxx} and [Mb] for (A) RV, (B) soleus, (C) gastrocnemius, and (D) EDL. Values are means ± SEM. Individual data points correspond to different strains or acclimation treatments. There were no significant correlations when using all strains and treatments (see text for additional details).

DISCUSSION

Genetic background has an impact on physiological traits in organisms such as aerobic capacity and endurance exercise performance (Bouchard et al., 1992; Lightfoot et al., 2001). This study sought to examine genetic background in the context of inbreeding and its impact on physiological responses to chronic hypoxia, from the whole animal to the intracellular level. The principle findings were that (1) inbred strains had a lower body mass than outbred strains, (2) hypoxia led to a decrease in body mass (except C3H, DBA and BSW) and increases in HET (except C3H, C, and BSW) and VO_{2max}, (3) there was a larger response of VO_{2max} to hypoxic acclimation in inbred than in outbred strains, and cost of exercise was higher in inbred strains, (4) the response of [Mb] to hypoxia was generally greater in magnitude in inbred than in outbred strains as indicated by the significant strain-treatment interactions, although pairwise comparisons indicated that the hypoxia induced changes in [Mb] were largely non-significant, and (5) that HET and VO_{2max} were positively correlated. Thus, while there is some support for the hypothesis that reduced phenotypic plasticity in inbred strains leads to a more variable response to hypoxia (in the [Mb] data), overall the results are not consistent with this view. However, the strong and consistent influence of inbreeding on body mass and VO_{2max} suggest an underlying consequence of genetic uniformity.

Acclimation to hypoxia resulted in a significantly lower body mass in all strains but two (inbred C3H and outbred BSW) relative to normoxic acclimated mice (Figure 1), a finding typical of altitude exposure observed in other mammals. For example, weight loss commonly occurs in the first weeks of exposure to moderate altitude in association with dehydration and a decrease in food intake attributed to loss of appetite (Hunter and Clegg, 1973). However, at extreme altitudes (above 5,400m), like that of the present study, weight loss may exceed that due to short term exposure and involve intestinal malabsorption or changes in fat metabolism and

total body water (Boyer and Blume, 1984; Hamad and Travis 2006). Inbreeding is also known to cause a reduction in body mass. Lynch (1977) found that in a line of mice derived from wild-caught progenitors, subsequent inbreeding over 6 generations resulted in a 40% depression of offspring body weight and a 12% depression of adult body weight. Similarly, over a 10-year period, inbreeding in ewes (*Ovis aries*) was increased up to 50%, which lead to as much as a 35% decrease in adult body mass (Ercanbrack and Knight, 1991). Other studies found similar effects of inbreeding on body mass in animals as diverse as fish (Gallardo and Neira, 2005) and wolves (Fredrickson and Hedrick, 2002), although the mechanistic basis of inbreeding induced loss of body mass is unknown.

After acclimation to hypoxia, HET was significantly higher in all strains except the C3H, C, and BSW mice (Figure 2) as is typically seen in mammals (Gonzalez et a., 1993; Calbet et al., 2003a; Calbet et al., 2003b). Overall, mice with higher body mass had lower HET values. Heavier mice often had difficulty running on the treadmill under hypoxia, as determined by a subjective assessment of running quality (rating of 1-3 from poor to excellent). Other studies have shown negative associations between body mass and duration of treadmill running, although the literature is still somewhat ambiguous on what, if any, relationship exists between body weight and activity levels in rodents (Barbato et al., 1998; Swallow et al., 1998; Turner et al., 2005). There was no significant effect of breeding on HET, and there also was no strain and treatment interaction that would suggest a more variable response of HET to hypoxia in inbred strains.

More markedly than HET, VO_{2max} increased in all strains following hypoxic acclimation (Figure 3). Acute or chronic exposure to hypoxia typically results in a decrease in VO_{2max} that is directly proportional to the reduction in ambient PO_2 (Shephard et al., 1988; Sutton et al., 1988; Cymerman et al., 1989; diPrampero and Ferretti, 1990; Green et al., 2000), which may be related

to circulation limitation (Favier et al., 1995) or a redistribution of cardiac output to nonexercising tissues (Calbet et al., 2003a). However, these prior studies differ from the current study in that they compare normoxic measurements of VO_{2max} to those measured under hypoxia. Here, all mice were exercised under hypoxic conditions, so the difference between the normoxic and hypoxic treatments reflects one means of assessing the capacity for acclimation to hypoxia (Young et al., 1985; Ferretti et al., 1997; Calbet et al., 2003a; Calbet et al., 2003b). Recovery of hypoxic VO_{2max} toward normoxic values following hypoxic acclimation has been reported by others and has been attributed to improved pulmonary gas exchange and ventilation as well as increases in blood hemoglobin concentration, hematocrit, capillarity, vasodilation, 2,3-bisphosphoglycerate (BPG), [Mb], and cardiac output (Gonzalaz et al., 1993; Hochachka et al., 1998; Calbet et al., 2003a).

Interestingly, inbred mice responded to hypoxic acclimation with a greater magnitude of change in VO_{2max} than outbred strains (Figure 3). This response may be due in part to the differences in body mass found between inbred and outbred strains. However, the analyses incorporated body mass as a covariate, and the effects of inbreeding on VO_{2max} were still significant. In addition, the cost of exercise was higher in inbred strains than outbred strains following hypoxic acclimation (Figure 4), meaning the inbred strains consumed more oxygen to maintain a given belt speed than the outbred strains. These results are consistent with a study by Hildner and Soulé (2004), who found a significant positive relationship between genetic variability and cost of burrowing among wild populations of pocket gophers (*Thomomys bottae*). Within each population there were two groups of subspecies, one with high genetic variability and one with low genetic variability. In two of the populations, whose subspecies had the greatest difference in genetic variability, there were 47% and 79% differences in cost between low and high genetic diversity subgroups. Thus, the populations with higher homozygosity had

higher costs of digging than the more heterozygous populations. Hildner and Soulé (2004) proposed that the observed differences were the result of inbreeding coupled with genetic drift in the small isolated populations, leading to lower burrowing efficiency (inbreeding depression) due to homozygosity for deleterious alleles.

VO_{2max} was positively correlated with HET, although the variance was high (Figure 7). Traditionally, VO_{2max} has been considered one of the most important predictors of exercise endurance (Costell, 1970; Bassett and Howley, 2000). This concept was first established in the classic study of Costell (1970), which found a strong correlation between VO_{2max} and 10-mile run times in humans. More recently this correlation has been demonstrated in rodents. VO_{2max} was 12% higher in rats (*Rattus norvegicus*, originally of N:NIH stock) artificially selected to be high capacity runners (HCR) than in those selected to be low capacity runners (LCR) during forced treadmill exercise following 7 generations of selection (Gonzalez et al. 2006). In this study, the difference was due almost entirely to a greater O_2 uptake and utilization by skeletal muscle in the HCR, while differences between lines in convective O_2 delivery to muscle by the cardiopulmonary system were minimal (Gonzalez et al., 2006). However, as the selection process continued to subsequent generations, VO_{2max} between lines diverged further as a consequence of an increased rate of tissue O_2 delivery. This resulted from an enhanced O_2 diffusing capacity in HCR rats. On the other hand, LCR VO_{2max} fell due to a decline in the ability to deliver O_2 to the exercising muscles. The diverging capacities resulted in an 8% increase in VO_{2max} in HCR from generation 7 to 15 and a 20% drop in LCR (Gonzalez et al. 2006). In a similar series of studies, mice (*Mus musculus*, originally of the Hsd:ICR strain) were selected for high voluntary wheel running (Swallow et al., 1998; Rezende et al., 2005; Rezende et al., 2006a; Rezende et al., 2006b). After 36 generations of selection, mice with a high capacity for wheel running had on average 10.7% higher VO_{2max} under hypoxia, 13.2% higher VO_{2max} under normoxia, and 20.8%

higher VO_{2max} under hyperoxia than non-selected mice. These studies emphasize the reliance of both forced and voluntary endurance on VO_{2max} in rodents, as the continued selection for increased endurance capacity in each case was followed by an increase in VO_{2max}.

Despite the positive correlation between VO_{2max} and HET (Figure 7), the variation in endurance between strains cannot be fully explained by VO_{2max}. Differences in other factors such as motivation to run could potentially explain some of the variation. For example, some of the mice preferred the shock of the grid over running, similar to observations in mice by both Lightfoot et al. (2001) and Swallow et al. (1998). In a study of interstrain variation in aerobic capacity among 10 strains of inbred mice, Lightfoot et al. (2001) observed wide variation in exercise capacity including two strains (A/J and AKR/J) that were "aggressively sedentary," and refused to exercise for more than a few minutes, preferring instead to attack the shock grid. The striking differences between strains were hypothesized to be due to genetic components that influence voluntary activity.

Since myoglobin is the final mediator of O_2 flux to the mitochondria, I proposed that [Mb] would reflect the efficiency of upstream components of the respiratory cascade in preserving O_2 supply to the muscle following acclimation to hypoxia. Prior studies have found that [Mb] does not change dramatically in response to chronic hypoxia. While studies in several species including humans, llama (*Lama glama*), and dog (*Canis familiaris*), have found small increases in [Mb] following hypoxia acclimation (Hurtado et al., 1937; Reynafarje, 1962; Cole, 1983; Garry et al., 2000; Hoppeler and Vogt, 2001; Ordway and Garry, 2004), others on humans have found no effect of hypoxia on [Mb] (Terrados et al., 1990; Masuda et al. 2001). Therefore, I postulated that the large hypoxic response of [Mb] previously observed in B6 and C mice (Sarkar, 2005) was a consequence of inbreeding and the associated reduction in phenotypic plasticity. That is, if one or more components of the respiratory system has limited plasticity due

to inbreeding, than other components such as myoglobin, may compensate by responding more dramatically. Thus, I predicted that inbred strains would have a large response to hypoxia that varied in magnitude and direction of change of [Mb], while outbred strains would be far less variable. While variation in [Mb] was generally greater among inbred strains (Figure 5 and 6), there were relatively few significantly different pairwise comparisons of normoxia and hypoxia. Further, the outbred strain SW had a similar magnitude of change to that of the B6 and C inbred strains in response to hypoxia, and some inbred strains displayed little change following hypoxic acclimation. Thus, the hypothesis that inbred strains would have larger changes in [Mb] following hypoxic acclimation and outbred strains would have smaller changes was not strongly supported.

Because VO_{2max} integrates the multiple components of the oxygen delivery system, including myoglobin, it can be used to represent a functional limitation of the cardiovascular system (Hill and Lupton, 1923; Bassett and Howley, 2000), and provide a physiological context for interpreting myoglobin concentration. However, among the muscle tissues tested, there was only a significant correlation of VO_{2max} and [Mb] in the RV of normoxic acclimated outbred mice (r^2=0.97; p=0.02), although the soleus was near significance (r^2= 0.47; p=0.059) for all mice and treatments (Figure 8). Likewise, there were limited significant correlations between HET and [Mb]. The only significant positive correlation was in the EDL of hypoxic acclimated mice, and in outbred mice there were negative correlations in the RV and gastrocnemius. However, because VO_{2max}, HET, and [Mb] were not each measured in the same mice, it was necessary to use mean values for each strain rather than data from individual mice. This reduced the statistical power of the analyses and may have resulted in undetected correlations. However, it is not necessarily unexpected that whole animal traits, such as HET and VO_{2max}, do not correlate with the concentration of a muscle-specific protein. In contrast, Duteil et al. (2004)

used nuclear magnetic resonance (NMR) techniques to reveal a correlation between skeletal muscle [Mb] and aerobic capacity of the same muscle, estimated from the recovery kinetics of phosphocreatine. Thus, such correlation may be difficult to detect unless aerobic function is examined in the same muscle in which [Mb] is measured.

In conclusion, hypoxia acclimation induced consistent changes across strains in body mass, HET, and VO_{2max}. Additionally, the changes in body mass and VO_{2max} were larger in inbred strains, although the mechanistic basis for these effects are unknown. Inbreeding did not appear to strongly influence HET or [Mb]. VO_{2max} was positively correlated with HET, supporting a role of aerobic capacity in determining endurance exercise capacity, but there was no clear relationship between myoglobin and either VO_{2max} or HET.

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